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JAMDA 18 (2017) 637.e1–637.e11

JAMDA

journal homepage: www.jamda.com

Original Study

Increased Myogenic and Protein Turnover Signaling in Skeletal Muscle of Chronic Obstructive Pulmonary Disease Patients With Sarcopenia

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Keywords:
Muscular atrophy
protein biosynthesis
proteolysis
myogenic differentiation

A B S T R A C T

Background: Sarcopenia was recently recognized as an independent condition by an *International Classification of Diseases, Tenth Revision, Clinical Modification* code, and is a frequently observed comorbidity in chronic obstructive pulmonary disease (COPD). Muscle mass is primarily dictated by the balance between protein degradation and synthesis, but their relative contribution to sarcopenia is unclear.
Objective: We aimed to assess potential differential molecular regulation of protein degradation and synthesis, as well as myogenesis, in the skeletal muscle of COPD patients with and without sarcopenia.
Methods: Muscle biopsies were obtained from the vastus lateralis muscle. Patients with COPD were clustered based on sarcopenia defined by low appendicular skeletal muscle mass index (nonsarcopenic COPD, n = 53; sarcopenic COPD, n = 39), and compared with healthy nonsarcopenic controls (n = 13). The mRNA and protein expression of regulators and mediators of ubiquitin-proteasome system (UPS), autophagy-lysosome system (autophagy), and protein synthesis were analyzed. Furthermore, mRNA expression of myogenesis markers was assessed.
Results: UPS signaling was unaltered, whereas indices of UPS regulation (eg, FOXO1 protein; p-FOXO3/FOXO3), autophagy signaling (eg, LC3BII/I; p-ULK1[Ser757]/ULK1), and protein synthesis signaling (eg, AKT1; p-GSK3B/GSK3B; p-4E-BP1/4E-BP1) were increased in COPD. These alterations were even more pronounced in COPD patients with sarcopenia (eg, FOXO1 protein; p-FOXO1/FOXO1; LC3BII/I; p-ULK(Ser555); p-AKT1/AKT1; AKT1; p-4E-BP1). Furthermore, myogenic signaling (eg, MYOG) was increased in COPD despite a concomitant increase of myostatin (MSTN) mRNA expression, with no difference between sarcopenic and nonsarcopenic COPD patients.
Conclusion: Together with elevated myogenic signaling, the increase in muscle protein turnover signaling in COPD, which is even more prominent in COPD patients with sarcopenia, reflects molecular alterations associated with muscle repair and remodeling.

M.L. and A.M.S. contributed equally to this article.
The authors declare no conflicts of interest.

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http://dx.doi.org/10.1016/j.jamda.2017.04.016
1525-8610/

Sarcopenia is defined as the loss of function in the presence of loss of muscle mass, and was recently recognized as an independent condition by an *International Classification of Diseases, Tenth Revision, Clinical Modification* code.[1] Apart from an age-associated decline in muscle mass, sarcopenia also encompasses the loss of muscle mass due to diseases such as chronic obstructive pulmonary disease (COPD).[2] In COPD patients, sarcopenia is a frequently observed comorbidity, affecting exercise capacity,[3,4] quality of life,[3] and survival.[5–7] Although muscle mass maintenance is considered to be primarily dictated by the balance between protein degradation and protein synthesis rates (ie, protein turnover), their relative contribution to imbalanced protein turnover during muscle mass loss remains unclear.

To provide a first insight into the potential drivers of this imbalance between muscle protein synthesis and breakdown in COPD patients, several studies assessed the molecular regulation of skeletal muscle protein turnover in COPD, and nearly all point to both increased protein degradation signaling[8–14] and increased protein synthesis signaling.[10,11,15] These findings are in accordance with the reported increase in whole body protein turnover rate in COPD as assessed by stable isotopes.[16] It was previously suggested that increased protein synthesis signaling in COPD may serve to maintain muscle mass in the presence of an elevated protein degradation rate.[11] However, such a compensatory mechanism to prevent muscle atrophy appears insufficient, because the prevalence of sarcopenia in COPD patients is high (ie, 12%–33%).[17–19] Although there are some data available on protein turnover signaling in sarcopenic COPD patients,[8,9,11] these studies are either limited in sample size or focus solely on either protein degradation or protein synthesis signaling. Furthermore, the role of myogenesis in muscle mass maintenance is frequently overlooked or incompletely assessed.

A comprehensive analysis of myogenic and protein turnover regulation in COPD patients with and without sarcopenia would provide further insight into the underlying skeletal muscle pathology, potentially providing new targets for intervention. In the present study, we therefore aimed to assess potential differential regulation of protein degradation and synthesis, as well as myogenesis, through analysis of an extensive panel of molecular regulators and mediators of myogenic and protein turnover signaling in the skeletal muscle of COPD patients with and without sarcopenia compared with healthy controls.

Materials and Methods

Study Design and Participants

The skeletal muscle molecular profiles of patients from 2 prospective cohort studies were analyzed. The study performed in Maastricht was registered at www.trialregister.nl as NTR1402, written informed consent was obtained from all participants, and the study was approved by the Maastricht University Medical Centre+ (Maastricht, the Netherlands) ethical review board (08-2-059). The study design was previously published.[20] The study performed in Golnik was registered at www.clinicaltrials.gov as NCT02550808, written informed consent was obtained from all participants, and the study was approved by the Slovenian National Medical Ethics Committee (Ljubljana, Slovenia). The study design was previously published.[21] Only data from baseline (ie, before pulmonary rehabilitation) measurements were used in the current study. All included patients were in a stable disease state, free from exacerbations in the 4 weeks before start of the study protocol.

Participants were excluded from the current analysis if the muscle biopsy or appendicular skeletal muscle mass index (ASMI) measurement was missing. Sarcopenia was solely defined according to cutoffs for ASMI (<7.23 kg/m^2 for men; <5.76 kg/m^2 for women)[22]; see Figure 1. One participant without COPD was classified as sarcopenic and was therefore excluded from the analyses, yielding a study population of 13 healthy controls and 92 COPD patients.

Pulmonary and Physical Function

Spirometry was used to obtain forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), and their ratio (FEV_1/FVC) according to the European Respiratory Society guidelines.[23] Patients were classified by disease severity based on Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage.[24] Physical function was assessed by peak load, determined by an incremental load cycling test as previously described.[25]

Anthropometry and Body Composition

Body mass index (BMI) was calculated as body mass/height^2 (kg/m^2). Whole body dual-energy x-ray absorptiometry (Hologic QDR Series Explorer bone densitometer; Hologic Inc, Marlborough, MA) was used to assess total and appendicular (ie, arms and legs) fat mass and fat-free mass. Fat-Free Mass Index (FFMI) was calculated as fat-free mass/height^2 (kg/m^2). ASMI was calculated as appendicular skeletal muscle mass/height^2 (kg/m^2).

Muscle Biopsy and Analyses

Biopsies were obtained from the vastus lateralis muscle of the dominant leg by needle biopsy, at least 20 hours after the last exercise test. Muscle tissue was snap-frozen in liquid nitrogen, and stored at −80°C. Tissue processing and molecular analyses were performed collectively.

Reverse Transcriptase–Quantitative Polymerase Chain Reaction and Western Blotting

Molecular analyses were performed in biopsies from all participants. After removal of technical outliers, which varied between different analyses, the total sample size for protein and mRNA targets included 10 to 13 controls, 50 to 53 nonsarcopenic patients with COPD, and 38 to 39 sarcopenic COPD patients.

Details on the procedures and the exact sample size per analyzed target are provided in the supplementary methods.

Statistics

Differences among nonsarcopenic patients with COPD, sarcopenic patients with COPD, and controls were tested by 1-way analysis of variance with Bonferroni post hoc comparisons in case of a significant group effect. Furthermore, differences between patients with COPD and controls were tested using independent *t* test. Relevant results of this comparison are presented in the figures. Analyses were performed using SPSS Statistics (version 22.0; IBM Corp, Armonk, NY). A *P* value less than .05 was considered statistically significant.

Results

Participant Characteristics

Based on gender-specific cutoffs for ASMI,[22] 39 COPD patients (42%) were sarcopenic, whereas 53 COPD patients (58%) and all control participants were nonsarcopenic. Patient characteristics are presented in Table 1. Although the sarcopenic COPD group contained slightly more men, groups did not differ significantly in sex distribution and age. BMI was lower in sarcopenic than in nonsarcopenic COPD patients, but controls did not differ from either COPD subgroup. Besides ASMI, FFMI was lower in sarcopenic COPD patients than in nonsarcopenic COPD

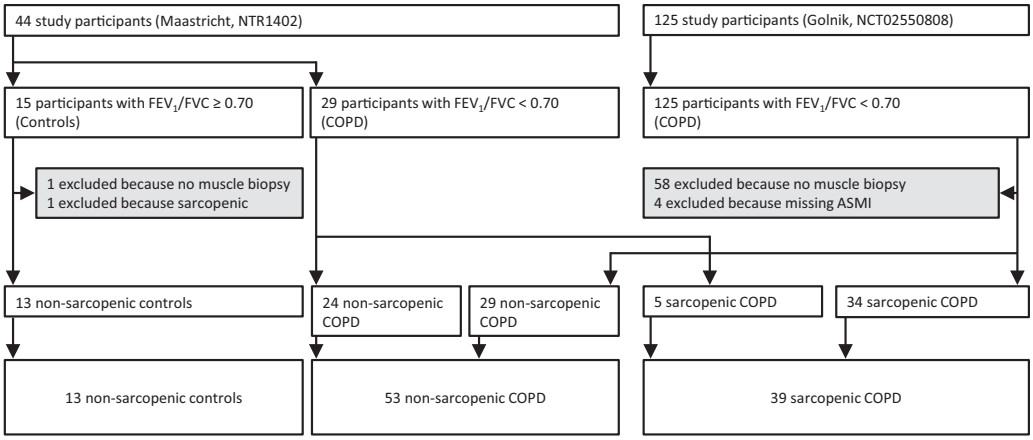

Fig. 1. Flow chart of participant selection and clustering. Sarcopenia: ASMI <7.23 kg/m^2 for men; <5.67 kg/m^2 for women.[22]

patients and controls. Furthermore, physical function was decreased in COPD patients compared with controls, and tended to be even further decreased in sarcopenic COPD patients compared with nonsarcopenic COPD patients. FEV_1/FVC ratio and FEV_1 (% predicted) were lower in COPD patients than in controls, and FEV_1 (% predicted) was even lower in sarcopenic than in nonsarcopenic COPD patients. Correspondingly, nonsarcopenic COPD patients most often had moderate (32%) to severe (45%) COPD, whereas sarcopenic COPD patients most often had severe (56%) to very severe (31%) COPD based on GOLD stage. Together, this shows that lung function is more impaired in sarcopenic than in nonsarcopenic COPD patients.

Protein Degradation Signaling

To assess potential differential regulation of skeletal muscle protein degradation signaling in COPD and sarcopenic COPD, markers of the 2 major proteolytic pathways were measured; that is, the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system (autophagy), which are in part regulated by forkhead box O (FOXO) transcription factors, including FOXO1 and FOXO3.

Table 1
General Characteristics

	Control, n = 13	COPD, No Sarcopenia, n = 53	COPD, Sarcopenia, n = 39	Overall *P* Value
Demographics				
Age, y	64.5 ± 5.4	64.0 ± 7.4	66.4 ± 8.4	NS
Sex, % male	53.8	60.4	74.4	NS
Body composition				
BMI, kg/cm^2	24.8 ± 3.4	26.7 ± 4.0	22.9 ± 2.8‡‡‡	**<.001**
ASMI, kg/cm^2	7.48 ± 1.01	7.42 ± 1.01	6.03 ± 0.85$^{**/‡‡‡}$	**<.001**
Female	6.52 ± 0.49	6.43 ± 0.61	4.98 ± 0.48$^{**/‡‡‡}$	**<.001**
Male	8.31 ± 0.37	8.06 ± 0.64	6.39 ± 0.61$^{**/‡‡‡}$	**<.001**
FFMI, kg/cm^2	17.9 ± 1.9	18.0 ± 2.5	15.9 ± 2.4$^{*/‡‡‡}$	**<.001**
Female	16.3 ± 1.4	15.9 ± 1.3	13.1 ± 1.1$^{**/‡‡‡}$	**<.001**
Male	19.2 ± 1.0	19.5 ± 2.0	16.8 ± 1.9$^{*/‡‡‡}$	**<.001**
Physical function				
Peak load, Watt	190.3 ± 62.8	73.4 ± 24.4**	58.1 ± 24.9$^{**/†}$	**<.001**
Female	136.0 ± 6.3	65.5 ± 19.2**	47.0 ± 19.5$^{**/‡}$	**<.001**
Male	236.9 ± 48.9	78.1 ± 26.1**	62.3 ± 25.7**	**<.001**
Lung function				
FEV_1, % predicted	113.5 ± 12.9	48.6 ± 18.3**	36.4 ± 12.9$^{**/‡‡}$	**<.001**
FEV_1/FVC, %	73.1 ± 4.9	39.5 ± 12.2**	34.5 ± 9.8$^{**/†}$	**<.001**
GOLD, %				**<.05**
1		6	0	
2		32	13	
3		45	56	
4		17	31	

Data presented as mean ± SD. NS: $P \geq .05$, Significant overall *P* values are indicated in bold, Significant post hoc comparisons after Bonferroni correction: $^{*}P < .05$, $^{**}P < .001$, compared with control group; $^{†}P < .10$, $^{‡}P < .05$, $^{‡‡}P < .01$, $^{‡‡‡}P < .001$, compared to nonsarcopenic COPD group.

Progressively Increased FOXO Signaling in COPD and Sarcopenic COPD

FOXO1 relative phosphorylation was unaltered in the skeletal muscle of nonsarcopenic COPD patients, but tended to be lower in sarcopenic COPD patients than in controls (Figure 2A). Remarkably, FOXO1 protein levels were higher in both nonsarcopenic and sarcopenic COPD patients than in controls, and were even further elevated in sarcopenic COPD patients compared with nonsarcopenic COPD patients (Figure 2C). This was paralleled by changes in *FOXO1* mRNA levels, which appeared higher in both nonsarcopenic and sarcopenic COPD patients, but reached significance only in sarcopenic COPD patients compared with controls (Figure 2D). Absolute FOXO1 inhibitory phosphorylation was concomitantly increased (Figure 2B), which may reflect an adaptation to compensate for the elevated FOXO1 expression. No group differences were found in FOXO3 mRNA and protein expression or absolute phosphorylation levels (Figure 2F–H); however, relative FOXO3 phosphorylation was lower in both nonsarcopenic and sarcopenic COPD patients than in controls (Figure 2E).

Unaltered UPS Signaling

In contrast to the apparent increase in FOXO activity, mRNA expression levels of the E3 ligases *TRIM63* (tripartite motif containing 63 [*MURF1*]) and *FBXO30* (F-box protein 30 [*MUSA1*]) were unaltered in the skeletal muscle of nonsarcopenic and sarcopenic COPD patients (Figure 3A, B). However, *FBXO21* (F-box protein 21 [*SMART*]) mRNA expression was higher in sarcopenic COPD patients than in controls (Figure 3C), whereas *FBXO32/MAFBX* (F-box protein 32 [*ATROGIN1*]) mRNA expression tended to be lower in sarcopenic COPD patients than in nonsarcopenic COPD patients (Figure 3D).

Progressively Increased Autophagy Signaling in COPD and Sarcopenic COPD

Among the FOXO target genes are the autophagy-related genes *MAP1LC3B* (*LC3B* [microtubule associated protein 1 light chain 3 beta]), *SQSTM1* (*P62* [sequestosome 1]), and *BECN1* (Beclin1).[26,27] In line with the unaltered *MURF1* and *MUSA1* mRNA levels, *LC3B* and *P62* mRNA expression remained unaltered (Figure 4D, F). *BECN1* mRNA expression, however, was higher in COPD patients than in controls (Figure 4H). Interestingly, LC3BI protein expression appeared higher in both nonsarcopenic and sarcopenic COPD patients but reached significance only in sarcopenic COPD patients compared with controls,

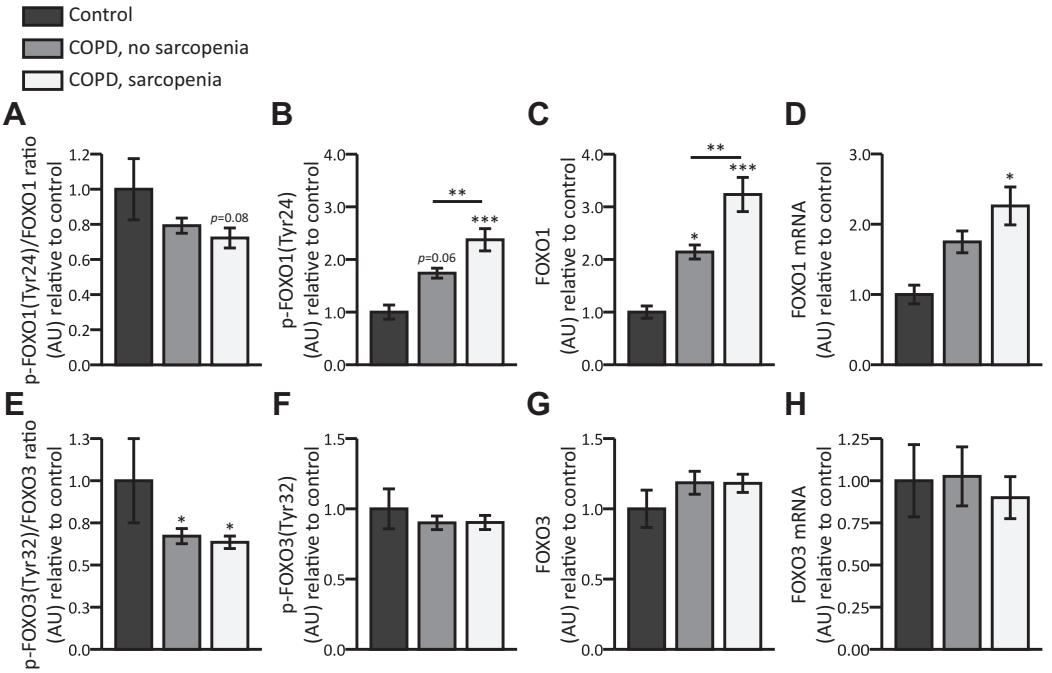

Fig. 2. FOXO regulation in COPD and sarcopenic COPD. (A) Relative FOXO1 phosphorylation at Tyr24. (B) Absolute FOXO1 phosphorylation at Tyr24. (C) FOXO1 protein expression. (D) FOXO1 mRNA expression. (E) Relative FOXO3 phosphorylation at Tyr32. (F) Absolute FOXO3 phosphorylation at Tyr32. (G) FOXO3 protein expression. (H) FOXO3 mRNA expression. Tyr, tyrosine. Values are means ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, compared with control group or between indicated groups.

whereas LC3BII levels were unaltered (Figure 4B, C). The resulting lower LC3BII/I ratio in sarcopenic COPD patients than in controls (Figure 4A) implies a change in autophagic flux, although no change in P62 protein expression was observed (Figure 4E). In line with the altered LC3BII/I ratio, BECN1 protein expression seemed to be higher in sarcopenic COPD patients (Figure 4G), but this did not reach statistical significance.

An important juncture in the initiation of autophagy is activation of unc-51 like autophagy activating kinase 1 (ULK1), which is regulated through phosphorylation by the upstream kinases; mechanistic target of rapamycin (MTOR) complex 1 (MTORC1) and AMPK. ULK1 relative inhibitory phosphorylation (serine [Ser]757) was lower in both nonsarcopenic and sarcopenic COPD patients than in controls (Figure 4I), whereas relative ULK1 activating phosphorylation (Ser555) was unaltered (Figure 4K). Furthermore, absolute ULK1 Ser757 phosphorylation, and ULK1 protein and mRNA expression were unaltered (Figure 4J, M, N), whereas absolute ULK1 Ser555 phosphorylation tended to be higher in sarcopenic COPD patients than in controls and nonsarcopenic COPD patients (Figure 4L).

Progressively Increased AKT/MTOR Signaling in COPD and Sarcopenic COPD

Coordination of protein degradation and protein synthesis signaling occurs via the PI3K/PKB (AKT serine/threonine kinase 1 [AKT1])/MTOR pathway. The relative phosphorylation of AKT1 was lower in the skeletal muscle of both nonsarcopenic and sarcopenic COPD patients than in controls, which tended to be more pronounced in sarcopenic COPD patients compared with nonsarcopenic COPD patients (Figure 5A). This reduced relative AKT1 phosphorylation did not result from a change in absolute AKT1 phosphorylation levels (Figure 5B) but from a higher AKT1 protein expression in nonsarcopenic and sarcopenic COPD patients than in controls, and in sarcopenic COPD patients compared with nonsarcopenic COPD patients (Figure 5C). Despite the change in AKT1 regulation, MTOR

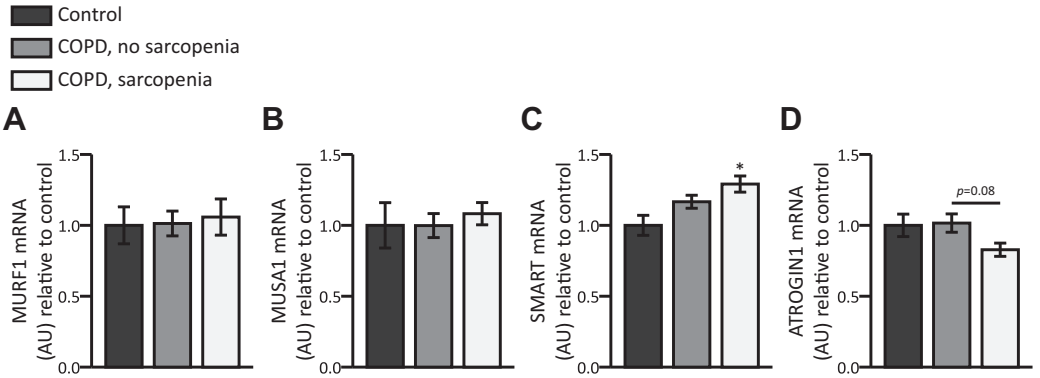

Fig. 3. mRNA expression of UPS markers in COPD and sarcopenic COPD. (A) MURF1 mRNA expression. (B) MUSA1 mRNA expression. (C) SMART mRNA expression. (D) ATROGIN1 mRNA expression. Values are means ± SEM. $^{*}P < .05$, compared with control group or between indicated groups.

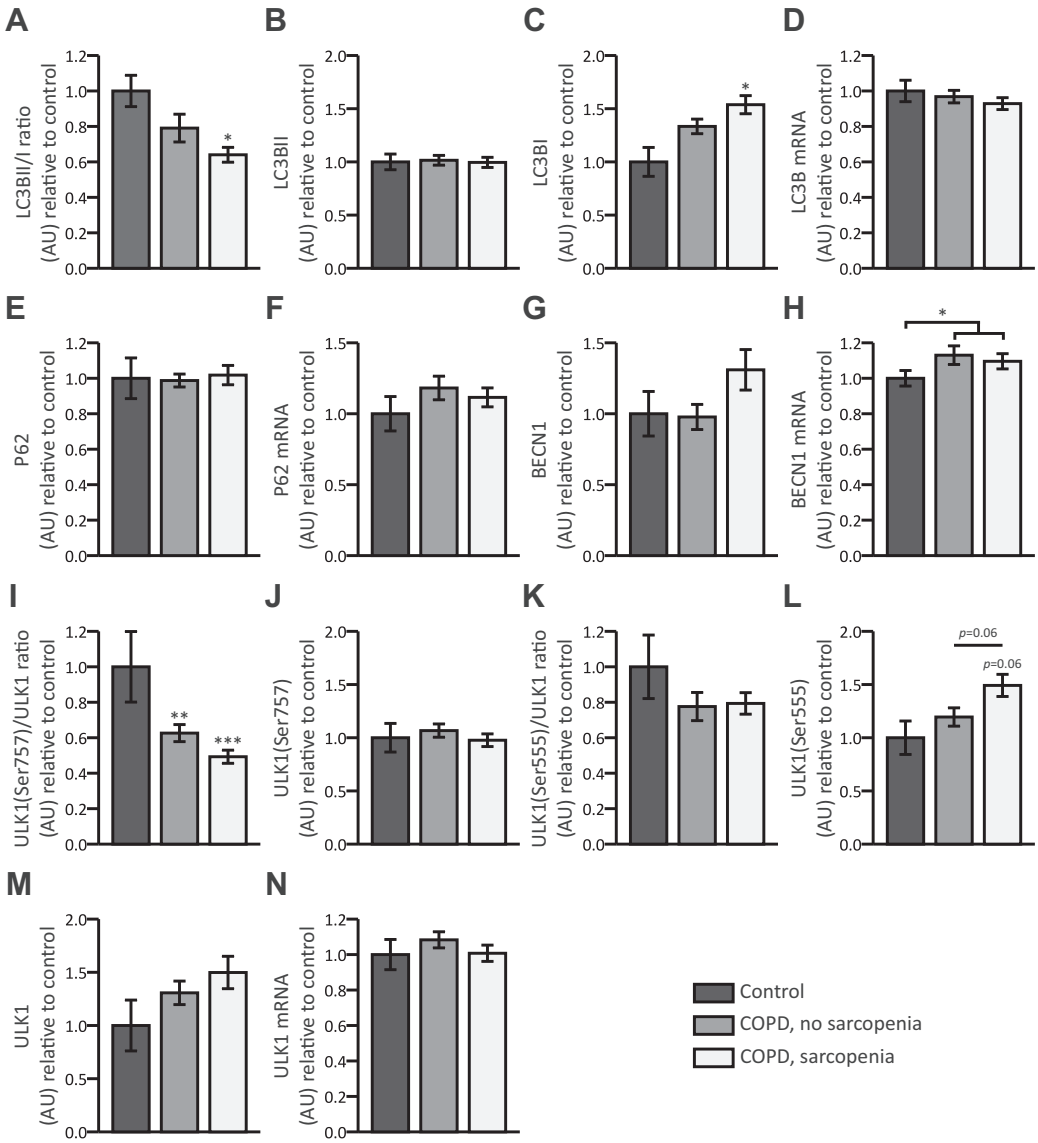

Fig. 4. Autophagic flux and autophagy initiation markers in COPD and sarcopenic COPD. (A) LC3BII/I protein ratio. (B) LC3BII protein expression. (C) LC3BI protein expression. (D) LC3B mRNA expression. (E) P62 protein expression. (F) P62 mRNA expression. (G) BECN1 protein expression. (H) BECN1 mRNA expression. (I) Relative ULK1 phosphorylation at Ser757. (J) Absolute ULK1 phosphorylation at Ser757. (K) Relative ULK1 phosphorylation at Ser555. (L) Absolute ULK1 phosphorylation at Ser555. (M) ULK1 protein expression. (N) ULK1 mRNA expression. Tyr, tyrosine. Values are means ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, compared with control group or between indicated groups.

phosphorylation (Figure 5D, E) and expression (Figure 5F) were unaltered. However, relative and absolute phosphorylation of the downstream AKT1 target glycogen synthase kinase 3 beta (GSK3B) were higher in sarcopenic COPD patients than in controls (Figure 5G–I), suggesting increased AKT1 activity.

Progressively Increased Protein Synthesis Signaling in COPD and Sarcopenic COPD

Although the MTORC1 downstream effector ribosomal protein S6 (RPS6) displayed unaltered relative phosphorylation (Figure 6A), both RPS6 protein expression and absolute phosphorylation were higher in COPD patients than in controls (Figure 6B, C). Furthermore, both relative and absolute phosphorylation of the translation repressor EIF4EBP1 (4E-BP1 [eukaryotic translation initiation factor 4E binding protein 1]) were higher in sarcopenic COPD patients than in controls (Figure 6D–F).

Increased Myogenic Signaling in COPD

Increased protein degradation and protein synthesis signaling in COPD and sarcopenic COPD suggest an increase in protein turnover. A similar pattern of molecular alterations has been observed during muscle remodeling after exercise.[28] We therefore asked if myogenesis, a process that is also implicated in muscle repair and remodeling, is increased in COPD and sarcopenic COPD patients as well. To this end, markers of satellite cell function were evaluated. The mRNA expression levels of the satellite cell marker paired box 7 (*PAX7*) and the proliferation markers proliferating cell nuclear antigen (*PCNA*) and cyclin D1 (*CCND1*) were unaltered in nonsarcopenic and sarcopenic COPD patients (Figure 7A–C). Furthermore, the mRNA expression of myogenic factor 5 (*MYF5*) was unaltered (Figure 7D). In contrast, myogenic differentiation 1 (*MYOD1*) and myogenin (*MYOG*) mRNA expression was higher in COPD patients than in controls (Figure 7E, F), and *CDH15* (cadherin 15 [*M-cadherin*]) mRNA expression showed a similar trend (Figure 7G). This

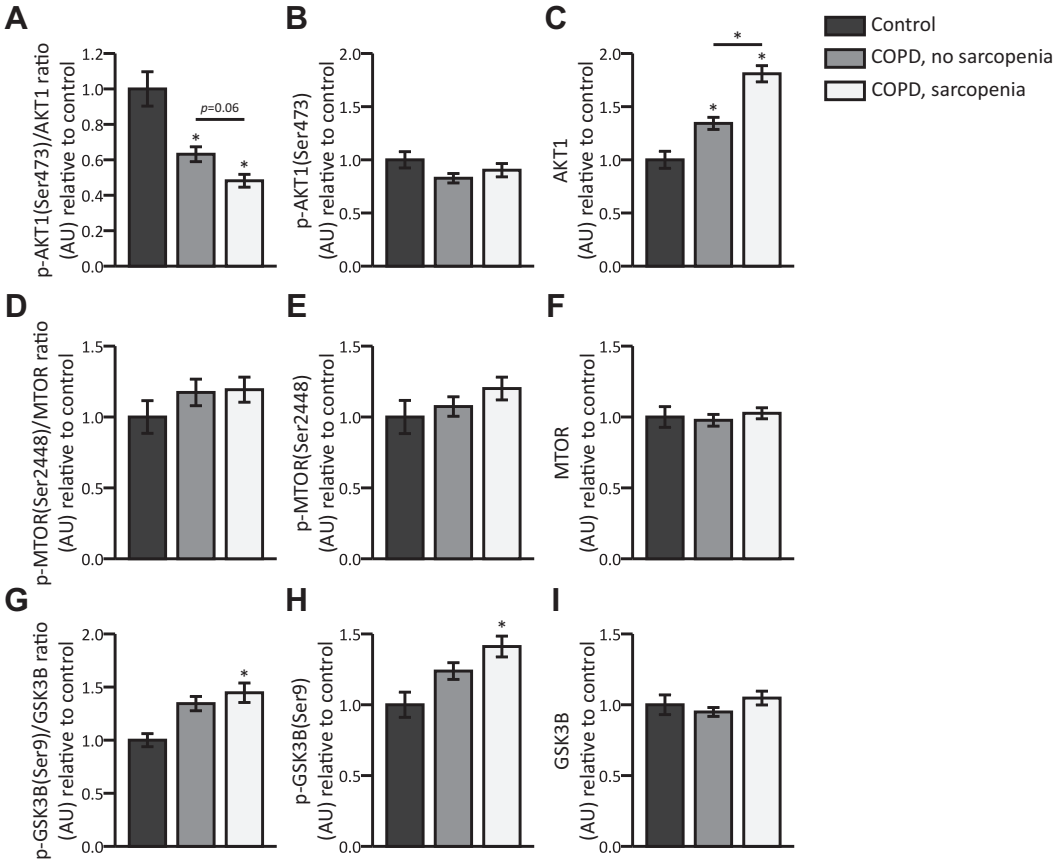

Fig. 5. AKT/MTOR signaling in COPD and sarcopenic COPD. (A) Relative AKT phosphorylation at Ser473. (B) Absolute AKT phosphorylation at Ser473. (C) AKT protein expression. (D) Relative MTOR phosphorylation at Ser2448. (E) Absolute MTOR phosphorylation at Ser2448. (F) MTOR protein expression. (G) Relative GSK3B phosphorylation at Ser9. (H) Absolute GSK3B phosphorylation at Ser9. (I) GSK3B protein expression. Tyr, tyrosine. Values are means ± SEM. *$P < .05$, compared with control group or between indicated groups.

implicates a sarcopenia-independent increase in the mRNA expression of muscle differentiation markers in COPD. In discordance with these findings, which suggested increased myogenesis, the mRNA expression of the myogenesis repressor *MSTN* (myostatin) was higher in the skeletal muscle of both nonsarcopenic and sarcopenic COPD patients than in controls (Figure 7H).

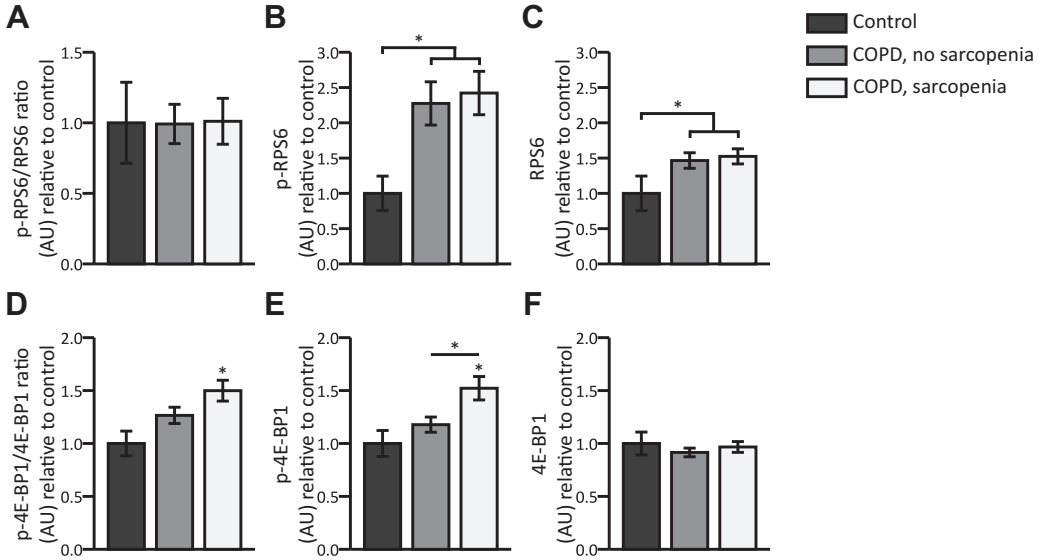

Fig. 6. Protein synthesis signaling in COPD and sarcopenic COPD. (A) Relative RPS6 phosphorylation. (B) Absolute RPS6 phosphorylation. (C) RPS6 protein expression. (D) Relative 4E-BP1 phosphorylation. (E) Absolute 4E-BP1 phosphorylation. (F) 4E-BP1 protein expression. Tyr, tyrosine. Values are means ± SEM. *$P < .05$, compared with control group or between indicated groups.

Discussion

By assessing a comprehensive panel of molecular regulators of muscle protein turnover, we demonstrate that both protein degradation and synthesis signaling are increased in the skeletal muscles of COPD patients, and extend previous findings by showing that these alterations are even more prominent in sarcopenic COPD patients. Furthermore, we show that myogenic differentiation signaling is increased in COPD, which is maintained, but not aggravated in sarcopenic COPD.

Several studies have addressed protein degradation and/or protein synthesis signaling in COPD or sarcopenic COPD.[8–14,29,30] However, due to differences in characteristics of the control groups, differences in disease severity of the COPD populations, and differences in body composition phenotyping, these studies cannot be directly compared or combined to extrapolate a conclusion on protein turnover regulation in COPD-related sarcopenia. In the present study, we therefore measured an extensive panel of molecular markers in skeletal muscle biopsies of a large, well-phenotyped COPD cohort to assess potential differential regulation of protein degradation and synthesis in the skeletal muscle of COPD patients with and without sarcopenia, and hint toward its potential implications for the level of protein turnover. Although we recognize that muscle protein degradation and synthesis rates were not measured in this study, given that this is a general limitation of many studies in humans, coordination between signaling and rates of turnover can be extrapolated from previous studies.[31]

Previous studies suggested increased FOXO signaling in the skeletal muscle of COPD patients and sarcopenic COPD patients.[11,29] Correspondingly, we find lower relative FOXO1 and FOXO3 inhibitory phosphorylation in COPD patients, implicating increased FOXO transcriptional activity. In line, the mRNA expression of the FOXO target *SMART* is higher in sarcopenic COPD patients than in controls. However, although previous reports showed increased ATROGIN1[8,9,13] and MURF1[11,29] expression in COPD and sarcopenic COPD, the present study shows no increase in the mRNA expression of *ATROGIN1*, *MURF1*, or *MUSA1*. In agreement with the study of Natanek et al,[30] *ATROGIN1* mRNA expression even tends to be lower in sarcopenic COPD patients than in nonsarcopenic COPD patients. Although the current data seem in disagreement with some of the previous literature, we and others have suggested that the increased expression of E3 ligases might be a feature of acute catabolic events rather than chronic muscle wasting,[30,32,33] the latter of which was studied in this cohort. We speculate that a recurring rise in UPS activity during repeated disease exacerbations results in an increased capacity for UPS induction, rendering the skeletal muscle more susceptible to catabolic triggers.[34,35] The higher FOXO1 protein and mRNA expression levels in muscle tissue of COPD patients and sarcopenic COPD patients may reflect such an increase in UPS inductive capacity. In this regard, the elevated absolute FOXO1 phosphorylation level may serve as a compensatory inhibition of (excess) protein degradation signaling in the stable disease phase.

In line with unaltered E3 ligase expression, the mRNA expression of autophagy-related FOXO target genes, *LC3B* and *P62*, is unchanged in COPD and sarcopenic COPD patients. Nonetheless, the higher *BECN1* mRNA expression level in COPD patients than in controls, and the apparent elevation in BECN1 protein expression in sarcopenic compared with nonsarcopenic COPD patients hint toward increased autophagy in COPD and sarcopenic COPD. It was previously shown that the number of autophagosomes is higher in the skeletal muscle of COPD patients and muscle-wasted COPD patients than in controls.[14,29] Although autophagic flux cannot be measured in vivo in humans, the lower LC3BII/I ratio in sarcopenic COPD patients compared with controls indicates that the level of autophagic flux is altered. The relative inhibitory phosphorylation (Ser757) of ULK1, an important juncture in autophagy initiation, is lower in COPD patients than in controls. Furthermore, the absolute Ser555 phosphorylation level tends to be higher in sarcopenic COPD patients than in controls or nonsarcopenic COPD patients. Together, this suggests that in the skeletal muscle of COPD patients, and even more prominent in sarcopenic COPD patients, autophagy is increased.

Higher absolute FOXO1 phosphorylation levels in COPD and sarcopenic COPD patients can result from increased AKT1 activity.

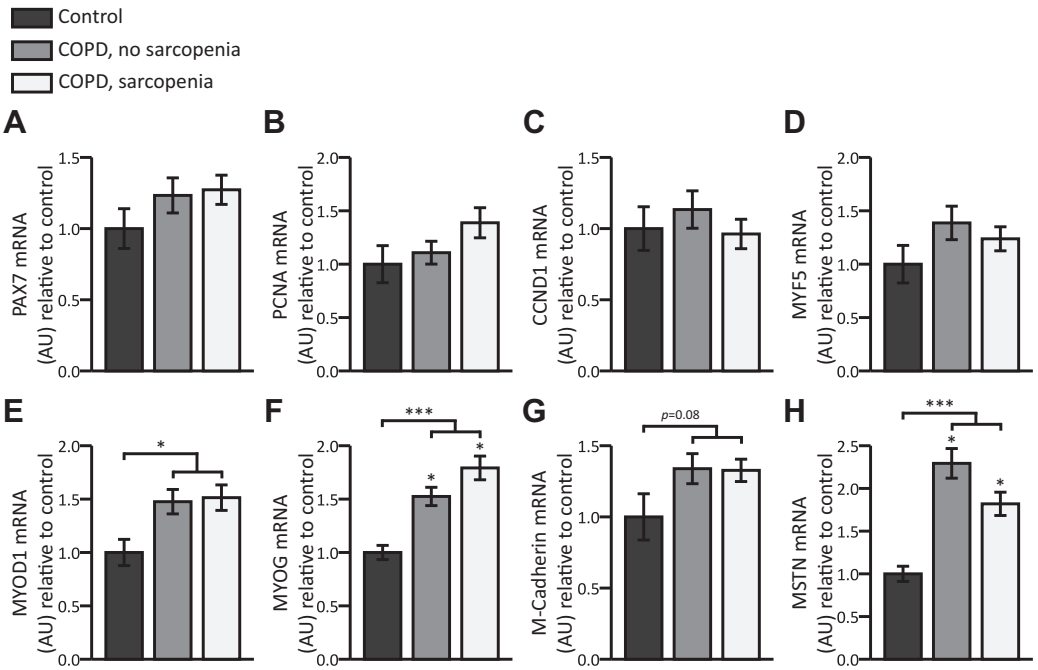

Fig. 7. mRNA expression of myogenic markers in COPD and sarcopenic COPD. (A) PAX7 mRNA expression. (B) PCNA mRNA expression. (C) CCND1 mRNA expression. (D) MYF5 mRNA expression. (E) MYOD1 mRNA expression. (F) MYOG mRNA expression. (G) M-cadherin mRNA expression. (H) MSTN mRNA expression. Values are means ± SEM. $^{*}P < .05$, $^{***}P < .001$, compared with control group.

However, several previous studies suggested a decreased AKT1 activity in COPD and sarcopenic COPD based on the relative AKT1 phosphorylation.[11,14] In line with this, we find lower relative AKT1 phosphorylation in nonsarcopenic COPD patients than in controls, and a trend toward a further decrease in sarcopenic COPD patients compared with nonsarcopenic COPD patients. Notably, the lower AKT1 relative phosphorylation is not reflective of altered absolute AKT1 phosphorylation, but rather of higher AKT1 protein expression. Moreover, phosphorylation levels of other downstream targets of AKT1 (ie, GSK3B, RPS6, 4E-BP1) suggest increased AKT1 activity. This stresses the need to not merely predict the enzymatic activity of phosphoproteins based on their relative phosphorylation state, but to also consider alterations in total protein abundance. Indeed, although the relative RPS6 phosphorylation remains unaltered, both the expression and activating phosphorylation level of RPS6 are higher in COPD patients than in controls, which is suggestive of increased mRNA translation capacity. Furthermore, the absolute inhibitory phosphorylation level of the negative regulators of translation initiation, GSK3B and 4E-BP1, is higher in sarcopenic COPD patients than in controls, implicating increased mRNA translation initiation. Together, these results show that the stimulatory signaling and capacity for protein synthesis is increased in the skeletal muscle of COPD patients in a stable disease state, particularly in those with sarcopenia.

Alterations in protein turnover are not necessarily accompanied by changes in muscle mass, and may serve several physiological processes, including muscle remodeling.[28] For example, recovery from muscle injury caused by eccentric exercise is accompanied by an increase in protein synthesis signaling.[36] Furthermore, a recent study showed an induction of autophagy during recovery from muscle injury and suggested a role for autophagy in muscle regeneration.[37] In the present study, muscle biopsies were taken at a basal state. Therefore, the increased autophagy and protein synthesis signaling suggest ongoing skeletal muscle remodeling in COPD and sarcopenic COPD patients, which may relate to muscle phenotypic alterations or damage repair. Indeed, previous studies showed damage of sarcomeres in the skeletal muscle of cachectic COPD patients.[29] Furthermore, recovery from muscle damaging eccentric exercise is accompanied by an increase in skeletal muscle satellite cell content,[38] which is indicative of myogenesis. Thèriault et al[39] reported unaltered satellite cell numbers in COPD patients with and without muscle wasting, whereas the number of central nuclei was higher in COPD patients with preserved muscle mass. Based on myogenic regulatory factor (MRF) protein expression, they suggested that satellite cells in COPD are under a sustained proliferative state. The current study, however, shows no change in the mRNA expression of *PAX7*, *PCNA*, and *CCND1*, suggesting no alteration in satellite cell numbers or proliferation in muscle of COPD patients. Furthermore, *MYF5* mRNA expression is unaltered, whereas the mRNA expression levels of *M-cadherin* and the MRFs, *MYOD1* and *MYOG*, are higher in COPD patients than in controls. Together, this points toward increased myogenic differentiation signaling in COPD, which is maintained, but not aggravated, in sarcopenic COPD.

Strikingly, but in line with previous research,[13] *MSTN* mRNA expression is higher in the skeletal muscle of COPD patients than in controls. MSTN is commonly known as a negative regulator of protein synthesis and myogenesis.[40] However, when considering the seemingly contradictory increase in expression of both *MSTN* and MRFs in COPD, it should be taken into account that the effect of MSTN is context dependent.[41,42] In vitro data showed that MSTN negatively affects myoblast proliferation and myogenic differentiation,[43,44] and in vivo MSTN deficiency elevates satellite cell numbers due to increased self-renewal.[41] As such, it seems obvious that proliferation is inhibited by MSTN. However, the inhibitory effect of MSTN on cell cycle progression,[41] often interpreted as detrimental for myogenesis, may actually facilitate terminal differentiation.[42,45] The higher *MSTN* and MRF mRNA expression in COPD may therefore be a physiological response to promote terminal differentiation as part of an increased demand for myonuclear accretion. Importantly, several studies suggest that temporal expression of MSTN controls the balance between proliferation and differentiation of satellite cells.[41,46,47] A sustained elevation of MSTN expression may therefore impair satellite cell activation and negatively regulate self-renewal,[41] eventually resulting in satellite cell senescence and depletion of the satellite cell pool.[42,48]

In conclusion, these results confirm increased protein degradation and synthesis signaling in the skeletal muscle of COPD patients compared with controls and show further stimulation of these processes in sarcopenic COPD patients compared with nonsarcopenic COPD patients. Together with the increase in myogenic signaling, this mirrors molecular alterations observed during muscle repair and remodeling (Figure 8).

Implications and Future Perspectives

Factors implicated in COPD-related sarcopenia, including oxidative stress and inflammation,[32] can result from and cause cellular damage,[49,50] and may drive repair and remodeling-like molecular alterations. Although these molecular alterations are likely necessary to facilitate repair of myofiber damage, a sustained increase in protein turnover regulation elevates the energetic costs of protein metabolism,[51] whereas a sustained increase in myogenic signaling could result in satellite cell

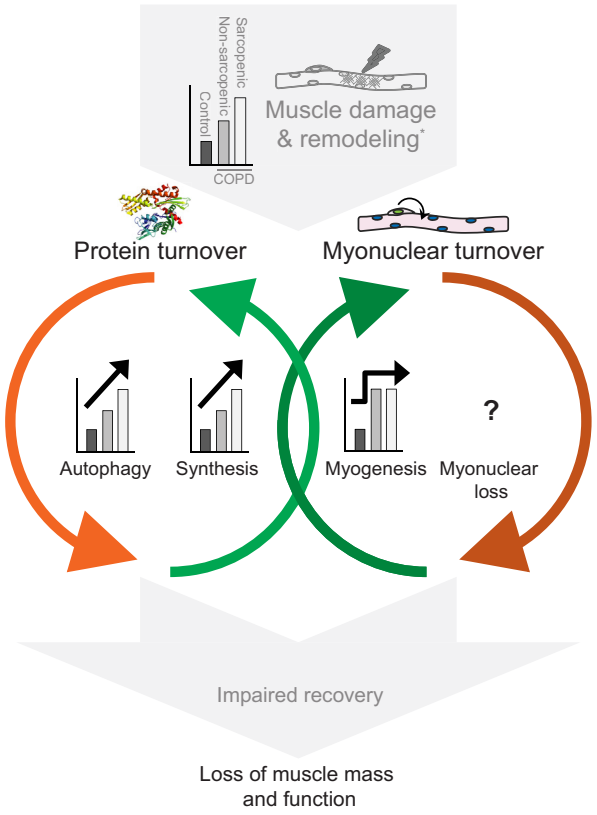

Fig. 8. Schematic presentation of sarcopenia-related molecular alterations in COPD. COPD-related factors leading to muscle damage and remodeling may drive the increase in myogenic and protein turnover signaling in COPD, as well as the even further increased protein turnover signaling in sarcopenic COPD, which may impair recovery after an exacerbation, leading to a progressive loss of muscle mass and function. Black text represents the regulation of indicated processes as measured in the current study, whereas gray text represents their associations and implications from previous literature. *Puig-Vilanova et al.[29]

depletion. This may not affect muscle mass substantially in a stable disease state, but it remains elusive to what extent this affects the coordination between anabolic signaling and the anabolic response required for muscle mass recovery after an exacerbation.

Future studies should therefore address the underlying mechanisms and pathophysiological relevance of repair and remodeling in the context of physiological responses to anabolic and catabolic stimuli in COPD patients, to elucidate the role of altered protein turnover and myogenic regulation in the development and treatment of COPD-related sarcopenia.

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Supplementary Methods

Reverse Transcriptase–Quantitative Polymerase Chain Reaction

Tissue was homogenized (Mini-BeadBeater; Biospec Products, Bartlesville, OK) in the presence of TRI-reagent (Sigma-Aldrich, St Louis, MO), and RNA was isolated by TRI-reagent/chloroform extraction and subsequently precipitated from the aqueous phase using glycogen-containing isopropanol. RNA was reconstituted in 30 μL RNA storage solution (AM7001; Ambion, Austin, TX) and stored at −80°C. RNA concentrations were measured spectrophotometrically using a Nanodrop UV-Vis spectrophotometer (ND-1000; Thermo Scientific, Waltham, MA). A total of 400 ng RNA was diluted in nuclease-free H_2O and reverse transcribed to cDNA using the Tetro cDNA synthesis kit (Bioline, Taunton, MA) according to the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) primers were designed based on Ensembl transcript sequences and ordered from Sigma Genosys (Zwijndrecht, the Netherlands), with primer details shown in Table E1. qPCR reactions contained Sensimix SYBR and ROX (GC Biotech, Alphen aan den Rijn, the Netherlands) and primer mix, and were run in a 384-well white opaque plate (Roche, Basel, Switzerland) on a LightCycler 480 system (Roche). Melting curves were analyzed to verify specificity of the amplification, and relative quantity of the targets was assessed by LinRegPCR software (v2014.8; bioinfo@amc.uva.nl).[1] Three reference genes (*RPLP0, B2M,* and *PPIA*) were used to calculate a GeNorm correction factor, which was used to normalize expression levels of the target genes.

Western Blot

Tissue was homogenized in 600 μL immunoprecipitation lysis buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 0.5% Nonidet P40, protease and phosphatase inhibitors [Roche]) with a Micro Tissue Homogenizer (Kimble-Kontes, Vineland, NJ). After homogenization, samples were incubated on a tube rotator at 4°C for 30 minutes and centrifuged at 14,000*g* at 4°C for 30 minutes. The supernatant was stored at −80°C until analysis. Total protein concentration was determined using a BCA Protein Assay kit (Pierce Biotechnology, Waltham, MA) according to the manufacturer's instructions. For Western blot analyses, 4× Laemmli sample buffer (0.25 M Tris-HCL pH 6.8, 8% [wt/vol] sodium dodecyl sulfate, 40% [vol/vol] glycerol, 0.4 M dithiothreitol, and 0.02% [wt/vol] Bromophenol Blue) was added and samples were heated to 100°C for 5 minutes; 10 μg protein was separated on a Criterion XT Precast 4-12% Bis-Tris gel (Bio-Rad, Hercules, CA) in XT MOPS running buffer (Bio-Rad) by gel electrophoresis. Proteins were transferred to a nitrocellulose membrane (Bio-Rad) by electroblotting at 100 V for 60 minutes in transfer buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol).

For total protein detection, the membrane was stained with PonceauS solution (0.2% PonceauS in 1% acetic acid; Sigma-Aldrich Chemie, Taufkirchen, Germany) and imaged using the Amersham imager 600RGB (GE Healthcare, Pittsburgh, PA). The membrane was blocked for 1 hour at room temperature (RT) in Tris-buffered saline with Tween20 (TBST; 20 mM Tris, 137 mM NaCl, 0.1% [vol/vol] Tween20, pH 7.6) containing 3% (wt/vol) nonfat dry milk (Campina, Eindhoven, the Netherlands). The membranes were washed in TBST, followed by overnight incubation at 4°C with primary antibody diluted in TBST with 3% bovine serum albumin or nonfat dry milk (Table E2). Membranes were incubated with a peroxidase-conjugated secondary antibody solution (#PI-1000, diluted 1:10,000 in blocking solution; Vector Laboratories, Burlingame, CA) for 1 hour at RT, and targets were visualized by chemiluminescence using Supersignal West PICO or FEMTO Chemiluminescent Substrate (Pierce Biotechnology) according to the manufacturer's instructions, and detected using the Amersham imager 600RGB. Signals were quantified with Image Quant software (Amersham). For analyses, samples from controls and patients with chronic obstructive pulmonary disease (COPD) were randomly distributed within and between blots, and reference samples were loaded onto all blots to correct for between-blot variation. Protein expression and phosphorylation levels were corrected for total protein content, and presented as a fold change compared with the control group.

Reference

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Table E1
qPCR Primer Sequences of Genes of Interest and Reference Genes

Target	n*	Source	Forward	Reverse
FOXO1	13/51/38	ENSG00000150907	CCTGGACATGCTCAGCAGACATC	TTGGGTCAGGCGGTTCATACC
FOXO3	13/51/38	ENSG00000118689	CACTGGTGCTAAGCAGGCCTCAT	ATGGCGTGGGATTCACAAAGGT
MURF1	13/51/38	ENSG00000158022	GCGAGGTGGCCCCATT	GATGGTCTGCACACGGTCATT
MUSA1	13/50/38	ENSG00000118496	AGTACTCCTGACAGCAGCAG	CATTGACACAGAAGTCAATCACTC
SMART	13/50/38	ENSG00000135108	AATTAATCTGAAAGGCACTGTGTC	TGAAGACAGAATGTCACAAACTG
ATROGIN1	13/51/38	ENSG00000156804	GAAGAAACTCTGCCAGTACCACTTC	CCCTTTGTCTGACAGAATTAATCG
LC3B	13/51/38	ENSG00000140941	ACCATGCCGTCGGAGAAGAC	TCTCGAATAAGTCGGACATCTTCTACTCT
P62	13/51/38	ENSG00000161011	GGTGCACCCCAATGTGATCT	CGCAGACGCTACACAAGTCG
BECN1	13/51/38	ENSG00000126581	AATGCAACCTTCCACATCTGGC	CCCAGCCTGAAGTTATTGATTGTGC
ULK1	13/50/38	ENSG00000177169	TGCCCGGTGTCCTGGTCCTC	CAGGGGCAGCAGTTGAGGCG
PAX7	13/50/38	ENSG00000009709	TACAGCACCACCGGCTACAGCGTG	CTGGCCGTACTGGCCGTACTGATAG
PCNA	13/50/38	ENSG00000132646	TCTGCAAGTGGAGAACTTGGAA	GAAGTTCAGGTACCTCAGTGCAA
CCND1	13/50/38	ENSG00000110092	ACCGCCTCACACGCTTCCTCT	GCCTGGCGCAGGCTTGACTC
MYF5	13/50/38	ENSG00000111049	TGCCCGAATGTAACAGTCCT	AGCAATCCAAGCTGGATAAGG
MYOD1	13/51/38	ENSG00000129152	CAATCCAAACCAGCGGTTGC	CCTCGATATAGCGGATGGCGT
MYOG	13/51/38	ENSG00000122180	TCAGCGCCAACCCAGG	GGTGAGGGAGTGCAGGTTGT
M-cadherin	13/50/38	ENSG00000129910	CCTGGACATCGCCGACTTCA	TGGGGTCACTATCTGCAGCC
MSTN	13/51/38	ENSG00000138379	AACCTTCCCAGGACCAGGAGAA	TGTCTGTTACCTTGACCTCTAAAAACGG
RPLP0	13/51/38	ENSG00000089157	TCTACAACCCTGAAGTGCTTGATATC	GCAGACAGACACTGGCAACATT
B2M	13/51/38	ENSG00000196262	CATCTGCACTGCCAAGACTGA	TTCATGCCTTCTTTCACTTTGC
PPIA	13/51/38	ENSG00000166710	CTGTGCTCGCGCTACTCTCTCTT	TGAGTAAACCTGAATCTTTGGAGTACGC

ATROGIN1 (FBXO32/MAFBX), F-box protein 32; B2M, beta-2-microglobulin; BECN1, Beclin1; CCND1, cyclin D1; FOXO1, forkhead box O1; FOXO3, forkhead box O3; LC3B (MAP1LC3B), microtubule-associated protein 1 light chain 3 beta; M-cadherin (CDH15), cadherin 15; MSTN, myostatin; MURF1 (TRIM63), tripartite motif containing 63; MUSA1 (FBXO30), F-box protein 30; MYF5, myogenic factor 5; MYOD1, myogenic differentiation 1; MYOG, myogenin; P62 (SQSTM1), sequestosome 1; PAX7, paired box 7; PCNA, proliferating cell nuclear antigen; PPIA, peptidylprolyl isomerase A; RPLP0, ribosomal protein lateral stalk subunit P0; SMART (FBXO21), F-box protein 21; ULK1, unc-51 like autophagy activating kinase 1.

*Sample size (controls/nonsarcopenic patients with COPD patients/sarcopenic patients with COPD).

Table E2
Primary Antibody List

Target	n*	Supplier	Product No.
FOXO1	12/52/38	Cell Signaling	2880
FOXO3	12/52/38	Cell Signaling	2497
FOXO1 (Tyr24)/FOXO3(Tyr32)	11/52/38	Cell Signaling	9464
LC3B	12/52/38	Cell Signaling	2775
P62	12/53/38	Cell Signaling	5114
BECN1	12/53/38	Novus Biologicals	NB110-87318SS
ULK1	12/53/38	Cell Signaling	8054
ULK1(Ser757)	12/53/38	Cell Signaling	6888
ULK1(Ser555)	12/53/38	Cell Signaling	5869
AKT1	12/53/39	Cell Signaling	2920
AKT(Ser473)	12/53/39	Cell Signaling	4060
MTOR	11/53/39	Cell Signaling	2983
MTOR(Ser2448)	11/53/39	Cell Signaling	2971
GSK3B	12/53/39	Cell Signaling	12456
GSK3B(Ser9)	12/53/39	Cell Signaling	9336
4E-BP1	12/53/39	Cell Signaling	9452
4E-BP1(Ser65)	12/53/39	Cell Signaling	9451
RPS6	10/53/39	Cell Signaling	2317
RPS6(Ser235/236)	10/53/39	Cell Signaling	4858

AKT1, AKT serine/threonine kinase 1; BECN1, Beclin1; FOXO1, forkhead box O1; FOXO3, forkhead box O3; GSK3B, glycogen synthase kinase 3 beta; *LC3B*, microtubule-associated protein 1 light chain 3 beta; MTOR, mechanistic target of rapamycin; P62, sequestosome 1; RPS6, ribosomal protein S6; Ser, Serine; Tyr, tyrosine; ULK1, unc-51 like autophagy activating kinase 1; 4E-BP1, eukaryotic translation initiation factor 4E binding protein 1.

*Sample size (controls/nonsarcopenic COPD patients/sarcopenic COPD patients).